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Epigenetic cell fate regulation of hepatic stellate cells

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Abstract

Research in the past three decades has identified key mediators and signaling mechanisms responsible for myofibroblastic transdifferentiation (MTD) of hepatic stellate cells (HSCs), the pivotal event in liver fibrogenesis. Yet, fundamental understanding of the MTD from the viewpoint of cell fate or lineage regulation, has been elusive. Recent studies using genetic cell fate mapping techniques demonstrate HSCs are derived from mesoderm and at least in part via septum transversum and mesothelium. HSCs express markers for different cell types derived from multipotent mesenchymal progenitors (e.g., neuron, smooth muscle cells, osteoblasts, adipocytes). A regulatory commonality between differentiation of adipocytes and that of HSCs is shown, and a shift from adipogenic to myogenic or neuronal phenotype characterizes HSC MTD. Central to this shift is a loss of expression of the master adipogenic regulator PPAR γ . Restored expression of PPAR γ and/or other adipogenic transcription factors (SREBP-1c, LXR α) reverses myofibroblastic HSCs to differentiated cells. In MTD, *Ppar γ* is epigenetically repressed by induction of the methyl-CpG binding protein MeCP2 and its enrichment to the promoter and PRC2/EZH2-facilitated H3K27 di/tri-methylation at the 3' exons. Blocking canonical Wnt signaling in myofibroblastic HSCs with the co-receptor antagonist Dickkopf-1, abrogates these epigenetic mechanisms, restores PPAR γ expression and HSC differentiation. Necdin, a melanoma antigen family protein, is identified as an upstream mediator for induction of the canonical Wnt10b and consequent *Ppar γ* repression and HSC MTD. The identified morphogen-induced epigenetic regulation of *Ppar γ* and HSC fate may serve as a novel target for manipulation of liver fibrosis and mesenchymal-epithelial interactions in liver regeneration.

Keywords

H3K27-methylation; MeCP2; mesenchymal progenitor; necdin; PPAR γ ; Wnt

Myofibroblastic transdifferentiation (MTD) of hepatic stellate cells (HSCs)

The essential role that myofibroblasts play in wound-healing responses of solid organs is well established, as is the concept of persistence of myofibroblasts in chronic injury leading to fibrosis ⁽¹⁾. Myofibroblasts are rare in normal tissues but are generated in response to trauma, inflammation, or infection by the trans-differentiation of resident cells. In the liver, myofibroblasts are generated by trans-differentiation of HSCs and fibroblasts ⁽²⁾. HSCs facilitate mesenchymal-epithelial interactions in the homeostasis of hepatocytes via the release of soluble factors such as TGF α ⁽³⁾, HGF ⁽⁴⁾, pleiotrophin ⁽⁵⁾, and epimorphin ⁽⁶⁾ and production of the components of the normal extracellular matrix (ECM) milieu of the perisinusoidal space ⁽⁷⁾. HSC MTD is the most extensively studied and best characterized mechanism for generation of myofibroblasts. In normal liver, HSCs are quiescent, lipid and retinoid-storing pericytes in the perisinusoidal space. In response to injury, HSCs undergo a dramatic phenotypic changes culminating in MTD which involves alterations in the expression of several hundred genes. The cell loses lipid and vitamin A storage and adopts a proinflammatory and profibrogenic phenotype associated with expression of the contractile protein smooth-muscle α -actin (α SMA) and abundant secretion of fibrillar collagens (collagen I and III) and the collagenase-inhibitor TIMP-1 ^(8;9). MTD is therefore a homeostatic response that ensures wound formation and contraction of a temporary scar. In acute injury, HSC-derived myofibroblasts are cleared in part by apoptosis ⁽¹⁰⁾, which is essential for subsequent repair and ECM remodeling as part of hepatic regeneration. By contrast, in chronic liver injury this process becomes dysregulated, resulting in persistence of myofibroblasts which proliferate and migrate along the liver sinusoids. Excessive secretion of cross-linked collagen and TIMP-1 promotes net collagen-rich ECM deposition which in a significant percentage of individuals progresses to severe fibrosis. There is therefore an urgent need to understand the molecular events that control the production, function and fate of myofibroblasts in chronic liver disease. To this end, the past three decades have witnessed a surge of investigative efforts which identified numerous mediators of HSC MTD. These mediators are largely classified into three categories ^(8;9): 1) soluble factors including cytokines, growth factors, hormones, and lipid mediators, etc; 2) oxidative stress arising within HSCs or from other cell types; and 3) chemical and physical changes in ECM milieu and components. Genetic factors that explain susceptibility or resistance to liver fibrosis are also of obvious importance and one such example is a recent discovery of a single nucleotide polymorphism of the Toll-like receptor 4 gene (c.1196C>T) which confers protection from fibrosis progression resulting from HCV infection ⁽¹¹⁾.

Origin of HSC

Despite active research on the molecular mechanism of HSC MTD, little attention has been paid to a fundamental question as to what this MTD process means from the standpoint of cell fate regulation. For this question to be addressed, we have to first understand the cell type of quiescent or “differentiated” HSCs. Adult liver HSCs express not only mesenchymal cell markers (e.g., vimentin), but also neural or neuronal markers including nestin, glial fibrillary acidic protein and p75 neurotrophin receptor (p75NTR) ⁽¹²⁾. Based on HSC phenotype with both mesenchymal and neural cell lineage markers being expressed, the neural crest was suggested to be the origin of HSCs. However, a genetic cell lineage analysis

using Wnt1-Cre and ROSA26 reporter mice failed to support this hypothesis⁽¹³⁾. Instead, this result favored the mesoderm origin of HSCs (Figure 1). Mesoderm-derived multipotent progenitor cells give rise to different cell types including osteoblasts, chondrocytes, smooth muscle cells, adipocytes, and neural cells (at least in culture), and HSCs express the markers of these cell types⁽¹²⁾. Indeed, careful morphologic studies in lower vertebrates by Wake, et al. more than two decades ago, suggested that vitamin A-storing mesenchymal cells including HSCs originate from the intermediate mesoderm and splanchnic mesoderm of the lateral mesoderm while fibroblasts are derived from the mesenchyme of the dorsal mesoderm and somatic mesoderm of the lateral mesoderm⁽¹⁴⁾. Thus it is plausible that HSCs originate from the multipotent mesenchymal progenitor cells derived from mesoderm. As hepatoblasts invade into the adjacent septum transversum mesenchyme around embryonic day 9.5, mesenchymal cells in the septum transversum are trapped between growing hepatoblasts and endothelial cells and appear to generate HSC⁽¹⁵⁾, raising a possibility that septum transversum mesenchyme is the HSC origin. This notion was recently validated by a genetic cell lineage analysis using ROSA26 reporter mice with Cre driven by the promoter of the early mesoderm-specific gene *MesP1*⁽¹⁶⁾ and a subsequent study which revealed that mesoderm-derived septum transversum mesenchyme and mesothelium as the origin of HSCs via the use of Wilms tumor 1 homolog-Cre/ROSA26 reporter mice⁽¹⁷⁾.

HSC fate regulation in mesenchymal lineages

Based on these findings on the HSC origin, one must examine cell fate regulation of HSC MTD within the context of mesenchymal lineages. It is noteworthy that “activated” HSCs upregulate expression of differentiation markers for the cell types derived from multipotent mesenchymal progenitors such as smooth muscle cells (α SMA)⁽¹⁸⁾, skeletal muscle cells (MyoD)⁽¹⁹⁾, and neurons (nestin, p75NTR, N-CAM)⁽²⁰⁻²²⁾ while the expression of PPAR γ , the master regulator of adipocyte differentiation, is reduced in activation⁽²³⁾. These results suggest that HSC MTD may be associated with a shift of the phenotype from adipogenic nature to myogenic and neural lineages. In fact, there are several similarities between differentiation of HSC and that of adipocytes. First, HSCs store substantial amounts of triglycerides besides retinyl esters like adipocytes⁽²⁴⁾. Secondly, both quiescent (differentiated) HSCs and adipocytes express type IV collagen whereas activated HSCs and preadipocytic fibroblasts express interstitial type I and III collagens. Thirdly, cytokines and growth factors (e.g., EGF/TGF α , TNF α , IL-1, TGF β , MCP-1, leptin) which are known to inhibit adipocyte differentiation, also activate HSCs. Lastly, adipocyte-specific genes such as leptin⁽²⁵⁾ and adiponectin⁽²⁶⁾ are expressed by HSCs. Based on these, we proposed that there must be a regulatory commonality between adipocyte and HSC differentiation⁽²³⁾ (Figure 2). Indeed, quiescent HSCs express putative adipogenic transcription factors such as C/EBP α , β , SREBP-1c, LXR α and PPAR γ , all of which are silenced/repressed with HSC activation⁽²⁷⁾. Treatment of activated HSCs with an adipocyte differentiation cocktail (isobutylmethylxanthine, dexamethasone, and insulin) or forced expression of PPAR γ or SREBP-1c reverses activated HSCs to differentiated cells with the restored ability to store vitamin A^(27;28). The specific effect of PPAR γ is supported by complete abrogation of the reversal effect by concomitant transduction of a dominant negative mutant of PPAR γ ⁽²⁸⁾.

Suppression of the activated phenotype of HSCs with synthetic or natural ligands for PPAR γ (23;29;30) is also consistent with the notion of adipogenic regulation being required for HSC differentiation. In addition, activated HSCs show increased expression and signaling of canonical Wnt mediated by Wnt10b and Wnt3a (31) which potentially suppress adipocyte differentiation via inhibition of the adipogenic transcription factors C/EBP α and PPAR γ (32;33) (Figure 2).

Epigenetic basis of HSC fate regulation

In HSC MTD, the genes that maintain the adipogenic or lipogenic nature of differentiated HSC such as PPAR γ , C/EBPs, SREBP-1c, and I κ B α , undergo coordinated transcriptional repression (23;28;34;35). In contrast, the genes that confer the myofibroblastic phenotype such as collagens I and III, TIMP-1, KLF6 and α SMA together with proinflammatory genes including ICAM-1, MCP1 and IL-6, are all transcriptionally activated (8;9). The simultaneous and well-coordinated nature of these genetic changes suggests an epigenetic blueprint for MTD and so-called “master transcriptional regulators” that interpret this blueprint to orchestrate conversion to the myofibroblastic phenotype. In fact, in order for multipotent mesenchymal progenitors to give rise to adipocytes via preadipocytic fibroblasts, tightly coordinated epigenetic regulation is required. This regulation allows a cluster of adipogenic genes to be activated by DNA de-methylation, H3K4 methylation, H3K9 de-methylation, HDAC downregulation, and histone acetylation, the changes which allow the formation of euchromatin for subsequent transcriptional activation of adipogenic genes such as *Ppar γ* (36). One of the most powerful silencing epigenetic signature which has to be eliminated for the initiation of the progenitor cell determination to pre-adipocytes is di- or tri-methylation of H3K27 by Polycomb repressive complex 2 (PRC2) whose methyltransferase activity is catalyzed by its constituent EZH2 (37). In fact, this histone modification and other negative chromatin modifications (H3K9 methylation, histone deacetylation) result in generation of heterochromatin and consequent silencing of adipogenic genes and de-differentiation of adipocytes to preadipocytic fibroblasts. As another compelling evidence for similar cell fate regulation between adipocytes and HSCs, we demonstrated similar epigenetic regulation exist for MTD of HSCs. First, loss of PPAR γ and I κ B α expression which occur during HSC MTD are prevented by the DNA methylation inhibitor 5-azadeoxycytidine (38). This observation led us to hypothesize that DNA CpG methylation and associated epigenetic regulation are responsible for acquisition of the myofibroblastic phenotype. In particular, we found the DNA methyl binding protein MeCP2 to be crucial for interpretation of the DNA CpG methylation “code” in activated HSCs. Our careful analysis of *Ppar γ* in activated HSCs has disclosed a novel epigenetic relay mechanism that is activated by diminution of miR132 resulting in overexpression of MeCP2 and its increased recruitment to *Ppar γ* promoter and alternative exons (39). This is associated with increased recruitment of the potent co-repressor HP1 α and H3K9 methylation for epigenetic repression of *Ppar γ* transcription at the 5' end. Further, MeCP2 overexpression results in increases in EZH2 expression and consequent H3K27 di-methylation at *Ppar γ* 3' exons (40) possibly via MeCP2-mediated epigenetic downregulation of miR137 which normally targets EZH2 as recently shown for neuron differentiation (40). In combination of these two effects on both 5' and 3' loci, the *Ppar γ* gene is silenced in myofibroblastic HSCs.

MeCP2 deficient myofibroblasts isolated from *MeCP2*^{-/-} mice have reduced EZH2 expression, H3K27 di-methylation, and H3K9 tri-methylation but increased H3K4 di-methylation at the *Pparγ* locus as compared to wild type cells. The treatment with the EZH2 inhibitor (3-deazaneoplanocin A) prevents activation of rat HSCs in culture and reduces the induction of α1(I) collagen and TIMP1 following acute CCl₄-mediated liver injury⁽³⁹⁾. Taken together, these findings demonstrate a previously unreported regulatory link of miR132-MeCP2-EZH2 in MTD of HSCs and suggest *in vivo* importance of this epigenetic regulation in liver fibrogenesis.

Necdin-Wnt pathway causes epigenetic repression of *Pparγ*

Blocking canonical Wnt signaling with the Wnt co-receptor antagonist Dickkopf-1 (Dkk-1) restores the expression of key adipogenic transcription factors, PPARγ and C/EBPα and reverses culture-activated HSCs to differentiated cells⁽³¹⁾. This reversal entails not only inhibition of Wnt target genes such as cyclin D1 and that of activation markers (type I collagen, αSMA, TGFβ), but also abrogation of endothelin-induced contraction⁽³¹⁾. Is this reversal effect mediated by epigenetic de-repression of *Pparγ*? That is most likely the case as the expression of Dkk-1 by an adenoviral vector in activated HSCs reduces MeCP2 enrichment to the *Pparγ* promoter and H3K27 di-methylation at the 3' exon locus⁽⁴¹⁾. Our recent study also identifies necdin as an upstream mediator for canonical Wnt induction in activated HSCs. Necdin is a maternally imprinted gene which encodes a 325 amino acid protein expressed in neurons, skeletal and smooth muscle cells, chondrocytes, pre-adipocytes, and skin fibroblasts and promotes neuronal and myogenic differentiation while inhibiting adipocyte differentiation⁽⁴²⁻⁴⁵⁾. Necdin deficiency in mice causes a neurobehavioral disorder similar to Prader-Willi syndrome⁽⁴⁶⁾. Our study demonstrates that necdin is selectively expressed in HSCs among different liver cell types in the rat and induced in HSC MTD both in culture and *in vivo* in animal models of liver fibrosis⁽⁴¹⁾. This activation-associated induction of necdin further supports our notion of adipogenic to neurogenic/myogenic shift of HSC phenotype in MTD. Necdin binds to the most proximal so-called “GN” box (multiple G clusters intervened with mono- or dinucleotides of A, T, or C) of the Wnt10b promoter and induces Wnt10b transcription⁽⁴¹⁾. Mutations of this binding site abrogate necdin-mediated Wnt10b promoter activity. Silencing of necdin with shRNA, inhibits Wnt10b expression, reduces MeCP2 enrichment to the *Pparγ* promoter and H3K27 di-methylation at the 3' exon locus, and reverses activated HSCs to differentiated cells in culture, and these effects of necdin silencing are prevented by addition of exogenous canonical Wnt3a⁽⁴¹⁾. Thus, these results identify a novel necdin-Wnt10b pathway which drives anti-adipogenic HSC MTD through epigenetic repression of PPARγ.

Implications in liver fibrosis and regeneration

Obviously, the disclosed epigenetic repression of the HSC differentiation gene *Pparγ*, may be considered as a new therapeutic target for liver fibrosis, so is the upstream necdin-Wnt pathway described above. However, both in liver regeneration and development, HSCs which presumably render the mesenchymal-epithelial interaction, have the phenotype of activated HSCs as characterized by reduced vitamin A content⁽⁴⁷⁾ and increased expression of αSMA and p75NTR^(21;48;49). Suppression of this “activated” or “undifferentiated”

phenotype of HSCs may in fact be detrimental as demonstrated in p75NTR deficient mice undergoing liver injury induced by plasminogen deficiency ⁽⁴⁹⁾. Deficiency of p75NTR results in insufficient hepatocyte regeneration despite suppressed collagen and α SMA expression ⁽⁴⁹⁾. Another recent study on rats undergoing 2-acetylaminofluorene administration and partial hepatectomy, also shows a drastically reduced number of activated HSCs in the periportal area by L-cysteine administration which is associated with a markedly attenuated over cell response ⁽⁵⁰⁾. In fact, it is believed that “activated” myofibroblastic HSCs provide the niche for regeneration and specification-promoting effects via release of soluble factors (e.g., HGF, FGF, pleiotrophin, epimorphin, keratinocyte growth factor, BMP4) in chronic liver injury and regeneration ^(21;51). Our finding on the involvement of the “morphogenic” *neccin*-Wnt pathway in HCS MTD via *Ppar γ* epigenetic repression, also suggests the potential role of this epigenetic regulation in the regenerative response as canonical Wnt pathway promotes mitogenic and cell fate responses (Figure 3). This important question remains to be addressed, so does the molecular mechanism underlying the MeCP2-EZH2 mediated epigenetic repression caused by canonical Wnt signaling (Figure 3). Further investigation in these areas should provide some insights into how we achieve optimal anti-fibrogenic effects while preserving or optimizing pro-regenerative responses via manipulation of HSC epigenetic cell fate regulation.

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Abbreviations

Dkk-1	Dickkopf-1
ECM	extracellular matrix
HGF	hepatocyte growth factor
H3K27	histone 3 lysine 27
HSCs	hepatic stellate cells
LXRα	liver X receptor α
MeCP2	methyl-CpG binding protein 2
MTD	myofibroblastic transdifferentiation
p75NTR	p75 neurotrophin receptor
PPARγ	peroxisome proliferator-activated receptor γ
PRC2	polycomb repressive complex 2
αSMA	smooth-muscle α -actin
SREBP-1c	sterol regulatory binding protein-1c
TGFα	transforming growth factor α
TIMP-1	tissue inhibitor of metalloproteinase-1

Figure 1.

Schematic diagram depicting two potential sources for the genesis of hepatic stellate cells (HSCs) in cell lineage development (neural crest, upper box and mesoderm-derived mesenchymal lineages, lower box). Results from genetic cell mapping analyses support the origin of HSCs from mesoderm and their differentiation within the lineage developments from multipotent mesenchymal progenitors (lower box).

Figure 2.

A regulatory commonality between differentiation of adipocytes and that of HSCs.

Adipogenic transcriptional regulation shown in upper half is also involved in differentiation (quiescence) of HSCs. Necdin-Wnt pathway antagonizes this regulation by suppressing PPAR γ .

Figure 3.

Epigenetic repression of *Pparγ* in HSC MTD. MeCP2 and EZH2 mediated epigenetic repression of *Pparγ* is depicted. In MTD, depletion of miR132 results in MeCP2 abundance and its recruitment to the *Pparγ* promoter while MeCP2-mediated upregulation of EZH2 causes H3K27 di- or tri-methylation at the 3' exons, preventing the recruitment of RNA polymerase II (RNAPII) and gene transcription. This repression results in loss of PPAR γ expression, a critical cause of HSC MTD. Activation of the necdin-Wnt pathway induces the MeCP2/EZH2-mediated epigenetic repression but its mechanisms are currently unknown. Whether or how this epigenetic *Pparγ* repression participates in pro-regenerative effects of activated HSCs, is also an important question.